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YrhB is a highly stable small protein with unique chaperone-like activity in *Escherichia coli* BL21(DE3)

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ABSTRACT

***Escherichia coli* YrhB (10.6 kDa) from strain BL21(DE3) that is commonly used for protein overexpression is a stable chaperone-like protein and indispensable for supporting the growth of BL21(DE3) at 48 °C but not defined as conventional heat shock protein (HSP). YrhB effectively prevented heat-induced aggregation of ribonucleotide synthetase (PurK). Without ATP, YrhB alone promoted *in vitro* refolding of uridine phosphorylase (UDP) and protected thermal denaturation of the refolded UDP. As a *cis*-acting fusion partner, YrhB also significantly reduced inclusion body formation of nine aggregation-prone heterologous proteins in BL21(DE3). Unlike conventional small HSPs, YrhB remained monomer under heat shock condition.**

1. Introduction

Owing to high production yield, low manufacturing cost, and well-established expression system, *Escherichia coli* has been used as a popular host to produce a wide variety of recombinant proteins, and moreover, the use of a lysogenic strain, *E. coli* BL21 is favorable for enhancing recombinant protein yield because it lacks OmpT protease and produces a low amount of acetate due to its constitutive transcription of the glyoxylate, gluconeogenesis, and anaplerotic pathways [1,2]. However, lack of appropriate folding-assistant proteins of *E. coli* causes aggregation and low yield of active recombinant proteins, mostly in the synthesis of eukaryotic foreign proteins. Therefore, there is always an urgent need for discovering novel folding assistant/enhancer proteins (e.g. chaperones or chaperone-like proteins as demonstrated in this study) from *E. coli*.

Molecular chaperones are a class of folding modulators that play a critical role in the conformational quality control of a wide range of proteins [3] and function under stress conditions as well as normal conditions [4]. Many chaperones are significantly upregulated in response to heat shock stressor leading to protein misfolding, and therefore the heat shock-responsive chaperones are named alternatively heat shock proteins [3,4]. Many chaperone or heat shock protein systems that are responsible for de novo folding in the cytoplasm of *E. coli* have been extensively studied, including trigger factor, DnaK/DnaJ/GrpE, GroEL/GroES complex, IbpA/B, Hsp31, Hsp33, ClpB, etc. [3,5]. Although the Hsp70/DnaK system is central and most effective in preventing protein aggregation [6], nearly 15% of newly synthesized polypeptides interact with DnaK, assisted by its co-chaperones in a co- or post-translational protein folding [7]. Furthermore, despite the abundance of GroEL/GroES chaperonin complexes, the majority of *E. coli* proteins are relatively small and folded without the aid of chaperonins [8], that is, the folding of more than 80% of *E. coli* proteins occurs independently of chaperonins [4,8,9]. Also, the information of other chaperone-like proteins used by *E. coli* to support de novo protein folding and manage stress-induced protein misfolding is not completely unveiled. Although over the past 30 years, there has been considerable progress in the fundamental understanding of protein folding and misfolding in *E. coli*, previously undiscovered molecular chaperones or chaperone-like proteins might exist [4,10,11].

Abbreviations: ADI, mycoplasmaarginine deiminase; AID, human activation induced cytidine deaminase; EGF, human epidermal growth factor; GAD, human glutamate decarboxylase; G-CSF, human granulocyte colony-stimulating factor; hFTN-L, human ferritin light chain; hIL-2, human interleukin-2; IPTG, isopropyl β-D-1-thiogalactopyranoside; LacZ, β-galactosidase; MOPS, 3-N-morpholino propanesulfonic acid; NACHT, cold autoinflammatory syndrome 1 protein Nacht domain; ONPG, ortho-nitrophenyl-β-D-galactopyranoside; ppGRN, human prepro-ghrelin; PurK, N5-carboxyaminoimidazole ribonucleotide synthetase; sHSP, small heat shock protein; UDP, uridine phosphorylase

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E. coli YrhB is a small intracellular protein with the molecular mass of 10.6 kDa, which has been designated yet as a hypothetical and uncharacterized protein. Through systematic proteome-wide analyses, we previously reported that the intracellular level of YrhB significantly increased only in response to heat shock [12]. In this study, it was found that YrhB plays an important role in supporting *E. coli* growth under heat shock condition. We also demonstrated the transcriptional regulation, stability, and the *trans*- or *cis*-acting chaperone-like activity of YrhB in promoting protein refolding and protecting protein aggregation/unfolding.

2. Materials and methods

2.1. Bacterial strains and cultivation

To construct the *yrh*B null mutant (*yrh*B$^-$), the TargeTron system (Sigma-Aldrich), which is designed for the specific gene disruption by inserting group II introns (i.e. catalytic RNAs), was used [13]. Briefly, the site-specific group II intron was inserted into the *yrh*B gene on the chromosomal DNA of *E. coli* BL21(DE3) using pACD4K-C vector (Sigma-Aldrich) that contains *yrh*B targeting sequence, group II introns, and chloramphenicol and kanamycin resistance genes.

Overnight inoculum cultures of wild type strain, *yrh*B$^-$, and *yrh*B$^-$[pHCE19(II)-YrhB] of *E. coli* BL21(DE3) [F$^-$ *omp*T *hsdS*$_B$(rB$^-$mB$^-$)] (Fig. S2) were prepared. The medium used for the growth of *yrh*B$^-$ mutant contained kanamycin (100 mg/l) and chloramphenicol (100 mg/l), while the medium used for the growth of *yrh*B$^-$[pHCE19(II)-YrhB] contained kanamycin (100 mg/l), chloramphenicol (100 mg/l), and ampicillin (100 mg/l). For the heat shock experiment, culture temperature was shifted to 48 °C when OD_{600} of the growing culture (50 ml) reached 0.5, and the bacterial growth of the mutant and wild type strains was monitored for an additional 5 h.

2.2. Expression vector construction and recombinant protein expression and purification

To construct the expression vector, pHCE19(II)-YrhB, the PCR product was inserted into the *EcoR*I–*Bam*HI site of plasmid pHCE19(II) (Bioleaders) (Fig. S2). The *yrh*B-negative mutant strain was transformed with pHCE19(II)-YrhB, after which the transformants were selected using LB-agar plates supplemented with ampicillin (100 mg/l), kanamycin (100 mg/l), and chloramphenicol (100 mg/l). The gene encoding the fusion protein, i.e. *N*-YrhB::heterologous protein-*C* and *E. coli* PurK (or UDP) gene were inserted into the *Nde*I–*Hin*dIII and the *Nde*I–*Xho*I site, respectively, of plasmid pT7-7 (or pETduet) to construct the expression vectors (Fig. S2). Detailed experimental procedures for protein expression and purification are described in Supplementary methods.

2.3. Thermal aggregation of YrhB-interactive protein, PurK

The *yrh*B-negative mutant strain was transformed with the expression vector pETDuet-PurK (Fig. S2) for the expression of PurK. At 4 h after the induction of gene expression at 37 °C, the bacterial culture was harvested, lysed, and PurK was purified according to the same procedures as described above. The purified PurK was properly diluted with 10 mM potassium phosphate buffer to adjust the concentration to 15 μM, after which the PurK solution was incubated for 30 min at 55 °C in the absence or presence of YrhB (50 μM) or bovine serum albumin (50 μM). Insoluble aggregate formation of PurK was monitored every 5 min through SDS–PAGE analysis.

2.4. In vitro refolding, thermal denaturation, and activity assay of UDP

UDP was expressed using pT7-7 plasmid vector (Fig. S2), purified, and totally denatured using 4 M guanidine hydrochloride and 50 mM MOPS (3-N-morpholino propanesulfonic acid) for 6 h at 37 °C. Refolding of UDP was carried out according to the previous report [14], and detailed procedures are described in Supplementary methods. For the thermal denaturation experiment of UDP, the refolded UDP (1.7 μM) was incubated for 2 h at 60 °C in the absence or presence of YrhB (1.7 μM). The UDP activity was photometically assayed according to the previous reports [15], and detailed procedures are described in Supplementary methods.

3. Results and discussion

3.1. YrhB plays an important role in protecting cells against heat shock-induced growth repression

Through proteome-wide analyses of soluble intracellular proteins, we previously reported multiple stressor(dithiothreitol, 2-hydroxyethyl disulfide, guanidine hydrochloride, and heat shock)-induced and up-regulated responses of the 80 *E. coli* BL21(DE3) proteins [12]. It was notable in this report that the intracellular quantity of the small hypothetical protein YrhB significantly increased only in response to heat shock (48 °C), indicating that the function of YrhB protein is presumed to be related to cellular activities under heat shock stress.

To evaluate the characteristics of YrhB under the heat shock condition, a *yrh*B null mutant strain of *E. coli* BL21(DE3) (*yrh*B$^-$)

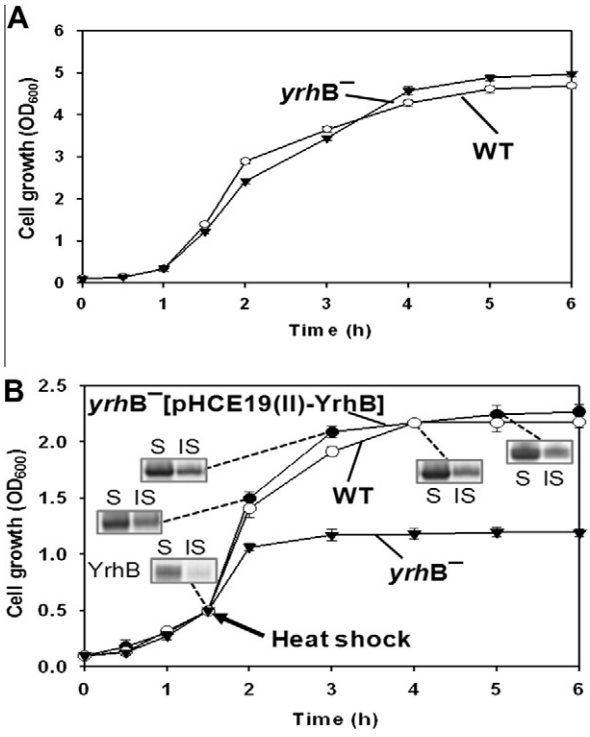

Fig. 1. Time-course variation in the bacterial culture growth that is estimated by absorbance (at 600 nm) of wild type strain (○), *yrh*B null mutant (*yrh*B$^-$) (▼), and *yrh*B$^-$[pHCE19(II)-YrhB] (●) of *E. coli* BL21(DE3). (A) Time-course growth of wild type strain and *yrh*B$^-$ mutant at 37 °C. (B) Time-course growth of the wild type strain, *yrh*B$^-$ mutant, and *yrh*B$^-$[pHCE19(II)-YrhB] at 48 °C. [The arrow indicates the time point of the culture temperature shift to 48 °C. The time-course variation in soluble (S) and insoluble (IS) fraction of YrhB that was constitutively expressed in *yrh*B null mutant (i.e. *yrh*B$^-$[pHCE19(II)-YrhB]) was also illustrated by the results of SDS–PAGE analyses.].

was constructed (see Experimental procedures for details). As shown in Fig. 1A, no significant difference in cell growth between the wild type and the *yrh*B-deficient mutant (*yrh*B$^-$) was observed when they were grown at 37 °C. However, the *yrh*B$^-$ strain was much more sensitive to heat shock than the wild type when exposed to high temperature (48 °C). As shown in Fig. 1B, the growth of the *yrh*B null mutant stopped within 1 h after the culture temperature increased to 48 °C, whereas under the same heat shock condition, the wild type continued to grow until the culture OD_{600} reached more than 2.0. (As presented in Fig. S1, the temperature 48 °C caused the most significant difference in cell growth between wild type and *yrh*B$^-$ strains, compared to the other growth temperatures.) Consequently, it seems that YrhB is important in supporting bacterial cell growth at high temperature. To confirm further if the heat-induced growth repression of *yrh*B$^-$ cells is due to the lack of YrhB, we examined the effect of plasmid-encoded production of YrhB (Fig. S2) on the growth of *yrh*B$^-$ cells under the heat shock condition. From Fig. 1B, it is noticeable that the *yrh*B$^-$ cells that constitutively expressed *yrh*B successfully overcame the heat shock, i.e. showed the same time-course growth as wild-type cells during the heat shock period, indicating that YrhB seems an essential protein for cell growth of *E. coli* under heat shock stress.

The main cellular activities to rescue heat-shocked cells involve protecting cells against protein aggregation/misfolding using primary chaperone systems [6,16] as well as activating in a coordinated manner heat inducible proteases to remove misfolded and aberrant proteins [11,17]. Therefore, the results of Fig. 1 suggest that YrhB plays a crucial role in sustaining cell growth at high culture temperature, probably through chaperone-like folding enhancer activity.

3.2. Transcription of yrhB is regulated by σ^{70}-dependent promoter

We cloned the transcription regulatory sequence (underlined sequence of Fig. S3) of *yrh*B (i.e. the entire sequence between two consecutive open reading frames of *yrh*A and *yrh*B), constructed plasmid expression vector (pYrhB-lacZα) (Fig. S2) containing the above upstream regulatory sequence of *yrh*B to transcribe α fragment of *lacZ* (*lacZ*α) and finally transformed *E. coli* DH5α [that constitutively expresses the other fragment (LacZω) of LacZ] with pYrhB-lacZα (see Supplementary methods). The measurement of time-course β-galactosidase activity at 37, 42, and 48 °C (Fig. S4) indicates that transcription of *yrh*B seems to be regulated by σ^{70}-dependent promoter, which also corresponds to the following previous results [18–21]: the transcription level of *yrh*B was not increased by the overexpression of *rpo*H [18,19] and was not upregulated by heat shock [20,21]. Consequently, it seems that YrhB cannot be defined as a traditional heat shock protein.

3.3. YrhB protects heat-induced aggregation in vitro, accelerates refolding, and protects thermal denaturation of refolded protein, showing chaperone-like function

We examined the effect of YrhB on *in vitro* thermal aggregation of purified PurK, N5-carboxyaminoimidazole ribonucleotide synthetase that is essential to purine biosynthesis (Fig. 2). [Through the comparative analyses of soluble proteomes of wild type, *yrh*B$^-$ mutant, and *yrh*B$^-$ mutant with plasmid-encoded production of YrhB under the same heat shock condition (48 °C), we found 23 proteins (including UDP) that showed more than 1.5-fold decrease in *yrh*B$^-$ mutant but more than 1.5-fold increase again in *yrh*B$^-$ mutant with plasmid-encoded production of YrhB. We also identified 5 proteins (including PurK) that totally disappeared in *yrh*B$^-$ mutant but appeared again when plasmid-encoded YrhB was constitutively expressed in *yrh*B$^-$ mutant (Fig. S5, Tables S1, S2).] The purified PurK solution (15 μM) was heated up to 55 °C, and the time-course variation of soluble fraction of PurK was analyzed through SDS–PAGE. From Fig. 2, PurK was rapidly aggregated at 55 °C without YrhB, whereas the aggregate formation noticeably reduced when the purified YrhB (50 μM) was present in the PurK solution at 55 °C. The reduced aggregation of PurK by YrhB was never reproduced by substituting YrhB with bovine serum albumin (BSA) (50 μM) (Fig. 2). Since the influence on thermal aggregation/denaturation at high temperature is regarded as a standard measure of chaperone activity [4,22], the result of Fig. 2 indicates that the uncharacterized protein YrhB has the chaperone-like function in protecting protein aggregation.

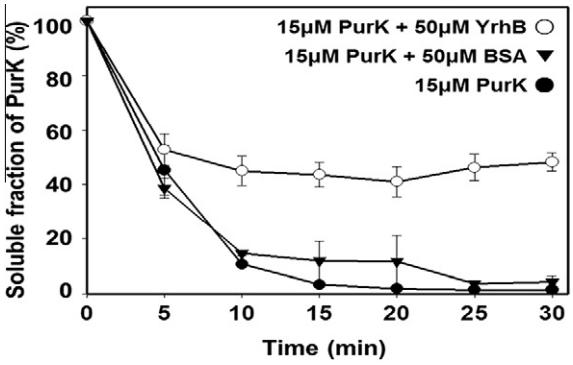

Fig. 2. Time-course variation of soluble fraction of purified PurK with and without YrhB (or BSA) in thermal aggregation at 55 °C. (Soluble and insoluble fractions of the centrifuged samples were analyzed using SDS–PAGE.)

We investigated the ability of YrhB to help a chemically-denatured endogenous enzyme, uridine phosphorylase (UDP) to restore its biological activity. UDP is an essential enzyme in nucleotide biosynthesis that catalyzes the phosphorolytic cleavage of the C-N glycosidic bond of uridine to ribose-1-phosphate and uracil. The denatured UDP solution (Materials and methods) was diluted with an excess volume of phosphate buffer for refolding [14] either in the presence or in absence of YrhB. The refolding of UDP (400 nM) was significantly more efficient in the presence of YrhB (200 nM) than without YrhB (Fig. 3A). Since DnaK/DnaJ/GrpE constitutes a main cellular machinery for efficiently renaturing thermally denatured proteins as well as protecting protein denaturation induced by heat shock [23], the effect of DnaK/DnaJ/GrpE on the refolding of UDP was also tested as a control. Fig. 3A shows that the effect of DnaK (400 nM) on refolding of UDP was almost the same as that of YrhB. (Without ATP, the addition of DnaK did not promote UDP refolding at all.) There does not seem to be a noticeable difference between YrhB and DnaK in promoting the UDP refolding, evidently indicating the folding enhancer activity of YrhB. Also, the UDP refolding with both YrhB (200 nM) and ATP (1 mM) did not show any noticeable difference compared to that with YrhB alone (without ATP), indicating that YrhB did not require ATP for its folding enhancer activity.

YrhB was also used to protect the thermal denaturation of the refolded UDP (1.7 μM). When temperature of the refolded UDP solution suddenly increased to 60 °C without YrhB, almost 90% of initial UDP activity disappeared within 2 h, whereas in the presence of YrhB, about 60% of the initial UDP activity was still detected even after 2 h (Fig. 3B). From the results of Figs. 2 and 3, it is concluded that although YrhB is not defined as a conventional heat shock protein, it has chaperone-like activity without requiring ATP.

3.4. The chaperone-like small protein YrhB shows high stability and unique property

We exposed the purified YrhB solution to heat shock condition (48 °C) for 10 h, and every hour the aggregate-free supernatant

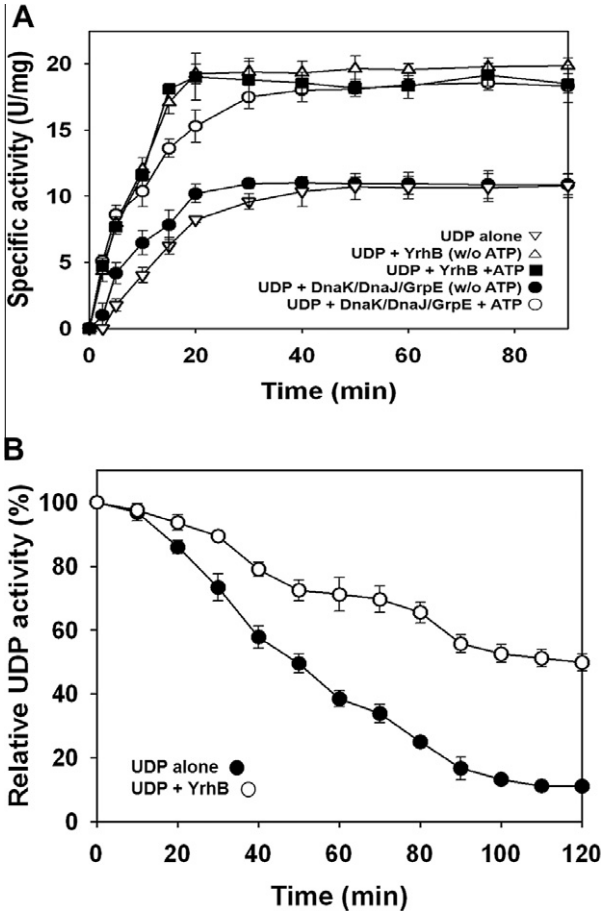

Fig. 3. Time-course variation of UDP activity during the refolding with and without YrhB, DnaK/DnaJ/GrpE, or ATP (A) and thermal denaturation of refolded UDP alone or with YrhB (B).

was subjected to non-denaturing gel electrophoresis (native PAGE). As shown in Fig. 4, almost all of the initial YrhB proteins remained soluble during the 10-h heat shock period, indicating that YrhB is extremely heat-resistant. [Fig. S6 also shows very high pH stability of YrhB in a wide range of pH (3–11).] Moreover, it is interesting to note that the initial monomer conformation of YrhB remained unchanged during the whole heat-shock period, which is a very unique characteristic, compared to conventional small heat shock protein (sHSP) family (e.g. IbpA, IbpB, etc.). Generally, sHSPs exist as large oligomeric structures where predominantly hydrophobic N-terminus domains are clustered in the assembled core

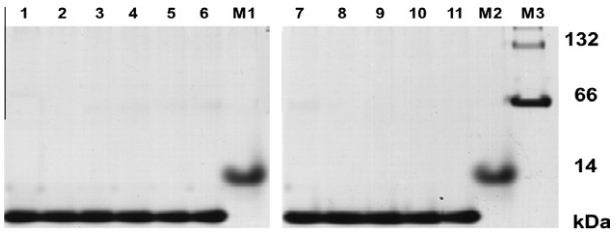

Fig. 4. Time-course analyses of non-denaturing PAGE of the purified YrhB (1 mg/ml) at heat shock temperature (48 °C) (To simultaneously load the 10 YrhB samples with 1 to 10-h heat shock period to a native PAGE gel, the 10 heat shock experiments started differently with 1 h interval.) [lane 1, YrhB before heat shock; lanes 2–11: YrhB samples with 1 to 10-h heat shock period, respectively; lanes M1 and M2: native PAGE molecular marker, α-lactalbumin from bovine milk (14 kDa); lane M3: native PAGE molecular markers, albumin mixtures from bovine serum, i.e. 66-kDa monomer and 132-kDa dimer].

[24–26]. However, multimeric complexes of sHSPs dissociate into the smaller subunits to expose hydrophobic binding sites at heat shock temperature and subsequently form high molecular weight complexes with substrate proteins to avoid protein aggregation [26–28]. Therefore, the small chaperone-like protein YrhB with extremely high thermal stability seems to have a very unique characteristic, because it keeps on maintaining a monomer conformation with undergoing neither any assembly nor disassembly under heat shock condition.

3.5. YrhB is used as a cis-acting fusion partner to express aggregation-prone heterologous proteins in BL21(DE3)

YrhB was used as N-terminus *cis*-element of aggregation-prone heterologous proteins that are expressed in *E. coli* BL21(DE3). Human epidermal growth factor (EGF), human prepro-ghrelin (ppGRN), human interleukin-2 (hIL-2), human activation induced cytidine deaminase (AID), human glutamate decarboxylase ($GAD_{448-585}$), Mycoplasma arginine deiminase (ADI), human granulocyte colony-stimulating factor (G-CSF), human ferritin light chain (hFTN-L), and cold autoinflammatory syndrome 1 protein Nacht domain (NACHT) have been reported to easily form inclusion bodies when directly expressed in *E. coli* [29]. We tried to synthesize the fusion protein, *N*-[YrhB]-[heterologous protein]-*C* using YrhB as an N-terminus *cis*-element. When directly expressed, the solubility of the aggregation-prone heterologous proteins in *E. coli* cytoplasm was negligible, i.e. only 4.0–8.3% (Fig. 5). However, the solubility of the aggregation-prone proteins dramatically increased up to the level of 25.4–96.5% when they were expressed with the N-terminus fusion of YrhB (Fig. 5), indicating that YrhB played as an effective *cis*-acting assistant protein that rescues the heterologous proteins from irreversible aggregation and inclusion body formation in *E. coli* cytoplasm. We previously reported the effect of a commercially available fusion tag [i.e. glutathione S transferase (GST)] on the solubility of nine heterologous proteins used in this study [30], showing that the effect of GST fusion is comparable to that of YrhB fusion. Generally, lower molecular mass of fusion partners is more advantageous in terms of yield of target protein, because the yield is determined after removing fusion partner although a large amount of fusion protein is synthesized by fusion partner with high molecular mass. YrhB (10.6 kDa) is much smaller than GST (26 kDa) and hence provides a significant advantage over GST.

Heterologous proteins	Directexpression	Fusion expression
	Solubility(%)	Solubility(%)
EGF	5.3 ± 2.5	25.4 ± 6.4
ppGRN	8.3 ± 2.1	96.5 ± 1.6
hIL-2	5.0 ± 2.2	87.9 ± 5.2
AID	8.0 ± 2.7	33.7 ± 4.1
$GAD_{448-585}$	4.7 ±1.5	43.6 ± 6.0
ADI	4.0 ± 1.0	88.0 ± 7.8
hFTN-L	5.0 ± 4.4	91.5 ± 4.8
G-CSF	4.7 ± 0.6	86.6 ± 5.0
NACHT	6.3 ± 2.1	32.8 ± 2.3

Fig. 5. The results of SDS–PAGE analyses showing the solubility (%) of various heterologous proteins that were expressed directly or expressed with the fusion of YrhB in *E. coli* cytoplasm. [Solubility (%) is defined as ratio of soluble fraction to total (soluble + insoluble) amount of the synthesized heterologous protein. (S and IS: soluble and insoluble fraction of the synthesized heterologous protein, respectively.)]

4. Conclusions

Although *E. coli*, especially the strain BL21 is a favorable host to produce a wide variety of recombinant proteins, lack of appropriate folding-assistant proteins causes aggregation and low yield of active heterologous proteins. Therefore, it is always of crucial importance to find novel *trans*- or *cis*-acting folding assistant chaperones or chaperone-like proteins. *E. coli* small protein YrhB that has been designated yet as hypothetical and uncharacterized protein was found noticeable through 2-DE-based analyses of soluble proteome of *E. coli* BL21(DE3), because YrhB was responsive only to heat shock stress among the 80 proteins, the intracellular quantity of which significantly increased even under severe stress conditions induced by four different stressors (dithiothreitol, 2-hydroxyethyl disulfide, guanidine hydrochloride, and heat shock) [12]. In this study, we also found that YrhB is indispensable for sustaining growth of *E. coli* BL21(DE3) at high culture temperature. A result of this study and other previous reports [18–21] show that the transcription of *yrh*B is not regulated by RpoH, indicating that YrhB cannot be defined as a traditional heat shock protein. This study reports that YrhB is extremely thermostable and keeps monomer conformation under heat shock condition unlike other small heat shock proteins that form multimeric or oligomeric structures. Since the same amount (45 μg) of soluble *E. coli* proteins was always used for 2-DE analyses [12], the reason for the significant increase of intracellular quantity of YrhB under heat shock stress seems to be due to high thermostability of YrhB to avoid heat-induced aggregation unlike many other *E. coli* proteins. (Only 45 out of 815 *E. coli* protein spots showed the increased fold change upon heat shock stress [12].) YrhB was able to dramatically reduce *in vitro* thermal aggregation of an essential *E. coli* enzyme PurK and effective in accelerating the refolding of another endogenous enzyme UDP. Its chaperone-like activity was also confirmed when YrhB was used to protect the thermal unfolding of the refolded UDP. Finally as a *cis*-acting element for the aggregation-prone heterologous proteins, YrhB effectively protected irreversible formation of inclusion bodies in *E. coli* BL21(DE3). It is concluded that although neither is thermosensitively transcribed nor belongs to the conventional small heat shock protein family, YrhB is a small and highly stable monomer protein with a unique chaperone-like function that enhances protein folding and protects proteins against *in vitro* or in vivo aggregation and denaturation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.02.051.

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